Virucidal properties of Orthosiphon stamineus against Herpes Simplex Virus Type 1 (HSV-1)

Nur Suhana Mohamad Ripim and Norefrina Shafinaz Md Nor*

School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.

Email: efrina@ukm.edu.my

ABSTRACT

Aims: Phytochemical analysis showed Orthosiphon stamineus (OS) possessed bioactive compounds with antiviral properties against Herpes Simplex Virus Type 1 (HSV-1). However, there isn’t any study reported so far on OS virucidal properties towards HSV-1. Thus, this study aims to investigate virucidal mechanism of OS aqueous extract that possibly acts as a potent entry inhibitor against HSV-1 infection.

Methodology and results: Virucidal attachment and penetration assays were done via plaque assay to investigate the virucidal anti-HSV-1 mechanism of OS. The aqueous extract of OS leaves (OSA) was found to reduce HSV-1 plaques in virucidal assays. Inhibitory effect by OSA was observed as early as 30 min after exposing OSA to HSV-1 in a concentration-dependent manner suggesting a direct anti-HSV-1 property of OSA. Further investigation of the stages in which OSA inhibits HSV-1 shows virions treated with OSA failed to attach onto the host cell which implicated a role of OSA in blocking HSV-1 attachment to its host. OSA was also found to reduce HSV-1 plaques in penetration assay. Further evaluation using transmission electron microscopy (TEM) on OSA treated virion showed defective HSV-1 virion without envelope and the remaining capsid was altered.

Conclusion, significance and impact of study: These findings concluded that Orthosiphon stamineus leaves extract have virucidal activity by disintegrating HSV-1 virion structure and interfering with the attachment and penetration of the virus into the host cell. Thus, through the new mechanism against HSV-1, OS has the potential to be further developed as an anti-HSV-1 agent.

Keywords: Ethnopharmacology, anti-HSV-1, virus attachment, virus penetration, plaque inhibition

INTRODUCTION

Orthosiphon stamineus Benth. (OS) is a well-known medicinal plant in Southeast Asia. This medicinal plant belongs to Lamiaceae family and has been widely utilised by the locals as herbal tea. In a recent study, OS hexane extract was proven to have the capability of elevating insulin expression and preventing glucotoxicity (Lee et al., 2015). Research carried out by Movahedi et al. (2015) showed that OS extracted using decoction method (aqueous) might have properties in preventing the development of liver cancer. In addition, various extractions of OS have been scientifically proven to exhibit a wide range of pharmacological properties as seen in a methanolic extract with antioxidant properties (Akowuah et al., 2004; Ho et al., 2010; Lim et al., 2013), antibacterial (Ho et al., 2010), antifungal (Hossain et al., 2008), an ethanolic extract with naphroprotective (Ramesh et al., 2014), antiangiogenicic (Al-Suede et al., 2012), and an aqueous extract with diuretic, uricosuric and hypouricemic properties (Olah et al., 2003; Arafat et al., 2008; Adam et al., 2009).

Phytochemical analysis of OS showed the presence of several well-known bioactive compounds especially from the flavonoid group (Akowuah et al., 2004; Loon et al., 2005; Yam et al., 2012; Hossain et al., 2015). Several independent studies showed some pure compounds from this group possess antiviral properties. For example, quercetin was proven to be active against HSV-1 and HSV-2 (Lyu et al., 2005) and reduced intracellular replication of HSV-1 (Kaul et al., 1985). However, there is no scientific evidence on the antiviral properties of aqueous extract from OS that has been reported to date. Since whole plant extracts have different dynamics when it comes to biological activity compared to pure compounds, the study on the effect of this extract is inevitable. It is also worth to note that there is the possibility that mixtures of compounds in the extract interact synergistically and therefore, exerting higher activity or antagonism (Krueger et al., 2009). Antiviral drugs with synergistic effects are especially advantageous in targeting virus with drug resistance issues, such as HSV-1.
HSV-1 infection, which has been a worldwide threat to public health, is endemic and highly contagious. For example, it is estimated that infection rates in neonates during delivery occurs in 10 out of 100 000 births globally (WHO, 2017). This infection is rare but may lead to neurological disability or death. In immune compromised people, HSV-1 may have more severe symptoms and frequent recurrences. HSV-1 lesions can be deeper and often longer compared to lesions in immune competent individuals with positive HSV-1 cultures in the lesions (Kalb et al., 1986). Severe recurrence of mucosal oral or genital lesions are much more common in immune compromised patients resulting in the spread of HSV-1 to other organs, causing pneumonitis, esophagitis, hepatitis and renal necrosis (Bacon et al., 2003). This enveloped virus is mainly transmitted by oral contact to cause infection in or around the mouth (oral herpes) and some proportion causes infection in genital areas. Genital herpes can be caused by either HSV-1 or HSV-2, which are very common, and data has shown that more than one in six people aged between 14 - 49 years have this health problem in the United States of America (CDC, 2017).

Since the discovery of acyclovir, a commercial anti-HSV-1 in 1978 and its structurally related compound penciclovir in 1980, these two drugs have been the main mode of treatment for HSV-1. Although acyclovir, penciclovir and their respective prodrugs are widely used and are effective in treating herpesvirus type 1 infections in immuno competent individuals, alternative treatment is important especially with the rise of resistance episodes in immuno compromised populations (Bacon et al., 2003; Price and Prichard, 2011). Both drugs have similar mechanisms of antiviral action against HSV-1. Both are analogues of natural deoxyguanosine and activation of the drugs need phosphorylation by viral thymidine kinase in HSV-1 virus-infected cell. Further phosphorylation of the drugs is done by cellular kinase which results in acyclovir or penciclovir triphosphate. Triphosphate drug will compete with natural nucleotides resulting to the termination of growing viral DNA chains (Elion, 1993). However, the mutant strain of HSV-1 has become resistant to antiviral activity of acyclovir. To make matters worse, the prevalence of acyclovir-resistant HSV is higher in severely immuno compromised individuals than immuno competent individuals (Englund et al., 1990). From the discovery of acyclovir 4 decades ago, only several drugs have gone beyond preliminary investigations. This includes Helicase-Primase inhibitors (HPIs) (Biswa et al., 2014), Docosanol, a fusion inhibitor (Antoine et al., 2013) and the latest BX795 that targets viral replication (Jaishankar et al., 2018). To date nothing has been described in depth about antivirals with virucidal activity. Continuous development of antiviral agents for this virus is necessary to provide better management strategies and to safeguard public health. Here, in this study, we show the potential of OS extract (OSA) to be developed as an alternative treatment against HSV-1 infection.

MATERIALS AND METHODS

Materials
Dried leaves of OS used in this study were supplied by Ladang Herba Ya’acob Berkat Enterprise, Bukit Katil, Malacca. Herpes Simplex Virus Type 1 (clinical strain) was obtained from stock culture of School of Biosciences and Biotechnology, Faculty of Science and Technology, UKM, Bangi, Selangor. African green monkey kidney cells (Vero) (ATCC CCL-81) were purchased from American Type Culture Collection and maintained with Dulbecco’s Modified Eagle’s Medium, DMEM (Sigma-Aldrich, USA) supplemented with 5% fetal bovine serum, FBS (Sigma-Aldrich, USA), non-essential amino acids (1×) and 100 μg/mL penicillin/streptomycin. Virus was propagated and quantified by a standard plaque assay in Vero cells (Nguyen et al., 2005).

Orthosiphon stamineus aqueous extraction (OSA)
Briefly, OSA extract was prepared by boiling 100 g of ground dried leaves with 1000 mL of deionized water for 2 h. The aqueous extract was cooled before being centrifuged at 1500 rpm for 5 min. Supernatant was collected and filtered using filter paper Whatman No.4 with diameter 125 mm (Whatman, UK). The filtered extract was frozen at −20 °C and lyophilised through freeze drying. In the test procedure, dried extract was diluted to produce working OSA concentration using serum-free medium.

Virucidal assay
Virucidal test was carried out based on methods described by Shogan et al. (2006). In this assay, two variables were used to determine virucidal activities which are time and OSA concentration. In the time variable assay, high titre of virus which can give high log reduction (10^6 PFU) was exposed to 0.39 mg/mL of OSA for 30, 60, and 90 min at 37 °C. In the concentration variable assay, 10^6 PFU was exposed to several concentrations of OSA (0.39, 0.2, 0.1 and 0.005 mg/mL) and incubated for 1 h at 37 °C. Virus that had been incubated with serum free DMEM was used as untreated control. After incubation, all tubes were diluted and titrated based on Nguyen et al. (2005). Each test was done in triplicates and the percentage of titre reduction was calculated by subtracting the average of virus titre of control with the average of treated sample (virus that was exposed to extract).

Attachment assay
The attachment assay used in this experiment was performed as described by de Logu et al. (2000) with slight modifications. Briefly, confluent Vero cells in 12-well plates were pre-chilled at 4 °C for 2 h. The medium was decanted, and cells were infected with 100 PFU HSV-1 in the presence of OSA at several concentrations, except for
untreated wells where cells were infected without OSA. Infected cells were further incubated for 3 h at 4 °C. After incubation, medium was aspirated and unadsorbed virus was removed with cold PBS several times. Overlay medium (DMEM+1% methylcellulose) was added and incubated for 48 h in 37 °C with 5% CO₂ before it was stained with crystal violet for plaque count. The percentage of inhibition of OSA of HSV-1 attachment was then calculated based on the formula below:

Percentage of plaque reduction:
(Average plaque count in untreated infected cells) – (Average plaque count in treated cells) (Average plaque count in untreated infected cells) × 100

Penetration assay

Penetration assay was done according to de Logu et al. (2000). Vero cells in monolayer were pre-chilled at 4 °C for an hour before being infected with 100 PFU HSV-1 and further incubated for 3 h to allow virus attachment. After incubation, virus inoculum was removed, and unadsorbed virus was washed with cold PBS two times. Then, OSA at several concentrations were added and further incubated at 37 °C with 5% CO₂ to allow maximum penetration of the virus. At 15 min intervals, infected cells were treated with PBS (pH3) for 1 min to inactivate virus that was unable to penetrate cell. Cold PBS was then used to wash the cells followed by DMEM without FBS. Overlay medium was added and the cells were further incubated for 48 h at 37 °C with 5% CO₂. The cells were stained, and the plaque formed was counted using the same formula as described in the attachment assay method.

Cells that were infected with HSV-1 at the same PFU without OSA treatment were used as the control. Each concentration was run in triplicates and the experiment was repeated three times.

Sample preparation for Transmission Electron Microscope (TEM) evaluation

The visualisation of HSV-1 particles was done using negative staining technique with Formvar grid as support film. Hydrophilic state of the grid was maintained beforehand by dropping 1% aqueous Alcian blue onto the grid for 5 min and then washing the grid with 5 drops of distilled water or until the rinse droplet became clear. Meanwhile, HSV-1 (10⁶ PFU) was incubated with 500µL of 0.39 mg/mL of OSA for 1 h at 37 °C with 5% CO₂ together with untreated virus sample tube (virus with DMEM only). After incubation, both samples were dropped onto the hydrophilic grid and allowed to spread and attach to the grid for 5 min. The grids were then washed using sterile distilled water for 30 sec. The samples were stained with 2% phosphotungstic acid (PTA) for 1 min and viewed using Tecnai FEI G2 Spirit (FEI, Hillsboro, OR, USA).

Statistical analysis

Statistical analysis was done using GraphPad PRISM with One Way Anova.

RESULTS

Total inhibition of HSV-1 plaque formation in virucidal assay

The evaluation of virucidal activity of OSA was done based on two parameters - different concentrations and durations of exposure (contact time) of OSA and HSV-1. In this study, exposure of 106 PFU HSV-1 to 0.39 mg/mL for the duration of 30 min inhibited 100% of plaque formation. Figure 1a shows exposure for 30, 60 and 90 min of 0.39 mg/mL OSA towards HSV-1, which caused a 100% reduction of viral titre. In the concentration variable assay, OSA as low as 0.10 mg/mL exhibited high antiviral activity where 80% of HSV-1 plaques were reduced. However, this virucidal activity of OSA was observed to be concentration-dependent with significant differences p<0.05. Antiviral activity was reduced significantly with reduction in concentration where the lowest concentration (0.05 mg/mL) administered for this assay showed 20% plaque reduction, the least inhibition efficacy observed (Figure 1b).

Figure 1: Virucidal screening of OSA: (a) Time variable assay where HSV-1 were exposed to OSA at variable time exposure (30, 60 and 90 min); (b) Concentration variable assay where different concentration of OSA were exposed to HSV-1 (0.05, 0.10, 0.20, 0.30 and 0.39 mg/mL)
Exposure to OSA reduced the attachment ability of HSV-1 to Vero cell

Further investigation was done to study the effect of direct OSA exposure to HSV-1 particle. We hypothesised that direct exposure of OSA will hinder HSV-1 initiation process of infection by preventing attachment of particle to the cell surface. In attachment assay, OSA was shown to effectively reduce HSV-1 plaque by as much as 97% at a concentration of 0.39 mg/mL. Observation result from this assay showed OSA activity was concentration-dependent where the effect of OSA decreased with decreasing concentration used for treatment. Statistical analyses showed significant difference between the activities of different concentration (p<0.05). The lowest concentration of OSA (0.013 mg/mL) showed the least plaque reduction where inhibition was less than 20%. Figure 2 shows inhibition of HSV-1 plaque is directly proportional to the concentration of OSA.

![Figure 2: Inhibition of OS in the attachment of HSV-1 virus by different concentrations of OSA (0.013, 0.025, 0.05, 0.1 and 0.39 mg/mL). Values represent ±SD from 3 independent experiments, calculated against untreated negative control (=100%); ****p < 0.05% (one-way ANOVA).](image-url)

(a) (b)
Figure 3: Percentage of HSV-1 plaque reduction in penetration assay which treated by OSA at six different concentrations (0.013, 0.025, 0.05, 0.1, 0.2 and 0.39 mg/mL) at different time exposure: (a) 15 min; (b) 30 min; (c) 60 min; (d) 1 h and 15 min; (e) 1 h 30 min; (f) 1 h and 45 min; (g) 2 h. Values represent ±SD from 3 independent experiments, calculated against untreated negative control (=100%); ****p < 0.05% (one-way ANOVA).
attachment and penetration assay, we had to host cells in the presence of OSA. In this study, rapid negative staining method using PTA was performed. PTA was chosen in this study because this type of stain outlines fringes or spikes of the HSV-1 envelope and does not cause positive effect of OSA in inhibiting initial process during HSV-1 infection. Microscopic evaluation using TEM was done to visualise and confirm whether the mechanism of OSA inhibition was due to morphological changes of HSV-1.

The complete lifecycle of a virus includes several critical steps; 1) attachment, 2) penetration and uncoating, 3) replication of genome and 4) assembly and release. Virus infection produces a circular zone called a plaque caused by viral lysis of the monolayer of infected cells during release of viral progeny. According to this principle, high number of plaques translates as a high rate of infection and low number of plaques means a low rate of successful infection. Virucidal refers to the effect of a chemical or agent that prevents infection due to physical changes to the virus and effectively ‘killing’ the particles. Here, we showed that OSA at 0.39 mg/mL reduced plaque formation (100% inhibition) by HSV-1 after 30 min minimum exposure in virucidal assay. Based on these results, in general, OSA anti-HSV-1 involves mechanism in inhibiting the process of attachment and penetration of HSV-1 into host cell. To further evaluate this, we then investigated the detrimental effect of OSA towards each process.

In the attachment assay, free HSV-1 virions were allowed to attach to host cells in the presence of OSA. In this assay, OSA was observed to impair HSV-1 attachment where results showed plaque formation was reduced by 97% at concentration of 0.39 mg/mL. Thus, from the result obtained, exposure of free virions to OSA resulted in neutralisation of viral infection. In the penetration assay, the second phase of the virus infection cycle was investigated. This assay investigated the ability of virions that had successfully attached to host cells to penetrate host cells after treatment with OSA. HSV-1 was allowed to attach to the cells in advance before administration of OSA. Allowance given to the virus to attach to host cell before being treated did not hinder OSA anti-viral efficiency significantly where plaque reduction by OSA was still highly efficient. This suggested that OSA antiviral activity affected HSV-1 penetration and might affect other proteins that are important in the subsequent viral cycle phase after entry. Nonetheless, treatment of OSA is most effective against free HSV-1 particles compared to treatment after virus was able to attach on the host cells.

For enveloped viruses, attachment followed by penetration into the host cell requires merger of viral envelope and target cell membrane. Based on our findings in attachment and penetration assay, we hypothesised that virucidal mechanism of OSA involved modification or disruption of HSV-1 structure mainly on envelope. To prove this hypothesis, we conducted direct observation towards treated HSV-1 using TEM. Detection of virus particles using TEM is known as a “catch-all” method where the device allows direct ocular analysis of whole virus particles and their surface morphology (Joshua et al., 2017). In this study, rapid negative staining method using PTA was performed. PTA was chosen in this study because this type of stain outlines fringes or spikes of the HSV-1 envelope and does not cause positive

**DISCUSSION**

Virucidal activity of OSA against HSV-1 is the focus of our study. Virucidal activity of OSA was evaluated in two different assays with different durations of exposure and concentrations as the parameters evaluated. High virucidal activity of OSA against HSV-1 in the screening assay provided us preliminary data to further investigate and understand the mechanism involved. Attachment and penetration assays were done to investigate the virucidal

**Figure 4:** PTA-stained of non-treated virus still intact. HSV-1 particles were observed to be intact. E = envelope, T = tegument, CM = capsomere (in box) and C = core (arrow) region of HSV-1. These three images of control were captured from different sample (triplicate). Figure 4a is PTA stained untreated HSV-1: a(i) Untreated HSV-1 at magnification 105,000×; a(ii) Untreated HSV-1 at magnification 135,000×; a(iii) Untreated HSV-1 at magnification 135,000×. Figure 4b PTA-stained of HSV-1 particles after being exposed to OSA for 60 min: b(i) OS treated of HSV-1 at magnification 43,000×; b(ii) OS treated of HSV-1 at magnification 220,000×; b(iii) OS treated of HSV-1 at magnification 220,000×.
staining (Goldsmith and Miller, 2009). Based on our observation, the appearance of a ‘fried egg’ structure, indicates the destruction of the envelope of HSV-1 particle, after being exposed to OSA. In addition, well-defined icosahedron capsid (Zhou et al., 2000) of virion was unable to be clearly defined along with the tegument. Research done by Steven and Spear (1997) revealed that the complex structure of an HSV-1 virion is composed of four distinct main structures which can be clearly visualized under an electron microscope. The four main morphological virion structures are the DNA core, capsid, protein rich tegument layer and envelope containing glycoproteins. HSV-1 possesses a lipid envelope which is lipophilic in nature and is sensitive to lipophilic-type chemicals (Klein and Deforest, 1983). HSV-1 envelope mediates attachment and penetration of the virus into the cells and provokes host immune responses (Whittle and Roizman, 2001). Capsid of HSV-1 is icosahedral with a T=16 shell (Caspar and Klug, 1962) made of 150 hexons and 12 pentons capsomere protein (Mettlenleiter, 2002). Capsid proteins are mainly protein in nature. The capsid’s essential role is mainly to protect viral nucleic acid and the facilitate nucleic acid entry when a suitable receptor on a host cell is encountered (Dohner et al., 2002). Virus is no longer infectious when the capsid loses its ability to protect viral genetic material from degradation in harsh environments (Baines and Roizman, 1992). The capsid is surrounded by tegument proteins. Tegment proteins play a variety of important roles including capsid transportation during entry and egress (Kelly et al., 2009). Thus, we have proven through observation under TEM that the inability of HSV-1 to attach and penetrate vero cells as shown by a high percentage of plaque reduction in the attachment and penetration assays was due to the disruption of HSV-1 structure that consists of important proteins for attachment and penetration. The HSV-1 envelope plays a very critical role in infection. Several researches have proven that the destruction of HSV-1 envelope has successfully inhibited viral infection. A protein analysis study was done by Terlizzi et al. (2016) to show that the interaction of cranberry extract (Oximacro®) with HSV-1 particle affects viral glycoprotein D. Their findings proved the virucidal activity of aqueous cranberry extract against HSV-1 was via interaction of the extract with the envelope protein, glycoprotein D. Similar results have been observed by Tiwari et al. (2010) in their research on anti-HSV activity of neem (Azadirachta indica L.) bark aqueous extract using viral glycoprotein through a cell-to-cell fusion assay. They showed that the anti-HSV-1 activity of the extract was due to its effect on viral particles which mediated the interaction with HSV-1 envelope glycoproteins, specifically glycoprotein D. Interaction of the extract with glycoprotein D inhibited the binding of HSV-1 to the target cell. Other than that, Western blot analysis of HSV-1 suspension, which was directly exposed to Myrothamnus flabellifolia extract also showed that the extract has an impact on envelope glycoprotein D. In their observation, glycoprotein D of non-treated HSV-1 particles migrated with an apparent molecular weight of 55 to 60 kD and incubation of HSV-1 (6.5×10^7 PFU/mL) with the extract led to reduction of the major gD band and to the oligomerisation of gD. Direct interaction of extract and HSV-1 caused blocking of viral attachment to the cell surface (Gesch et al., 2011). Nonetheless, none of these studies have shown the effect of these plant extracts through direct observation of the virion after treatment. Here, we show the images of HSV-1 affected by OSA to prove the ability of OSA in destroying HSV-1 virions that leads to a loss of pathogenicity.

CONCLUSION

In conclusion, our study showed that OSA has high antiviral activity against HSV-1 by a virucidal mechanism that prevents the host cell attachment and penetration of HSV-1. This mechanism is different from commercial anti-HSV-1 drug acyclovir making it a good candidate for development as an anti-HSV-1 agent especially against resistant strains.

ACKNOWLEDGEMENT

We would like to thank the School of Biosciences and Biotechnology, Faculty of Science and Technology, UKM, for the facilities and infrastructure. We would like to thank UKM for the funding of this research through research grant (GGPM-2012-108).

REFERENCES


decades of antiviral therapy. Clinical Microbiology Reviews 16(1), 14-128.


CDC. (2017). Genital Herpes - CDC Fact Sheet (Detailed). Clifton Road Atlanta, USA. CDC-INFO.


Pharmaceutical and Biomedical Analysis 33(1), 117-123.


